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Amino-Terminal Substitutions in the CCR5 Coreceptor Impair gp120 Binding and Human Immunodeficiency Virus Type 1 Entry

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The CC-chemokine receptor CCR5 is required for the efficient fusion of macrophage (M)-tropic human immunodeficiency virus type 1 (HIV-1) strains with the plasma membrane of $CD4^+$ cells and interacts directly with the viral surface glycoprotein gp120. Although receptor chimera studies have provided useful information, the domains of CCR5 that function for HIV-1 entry, including the site of gp120 interaction, have not been unambiguously identified. Here, we use site-directed, alanine-scanning mutagenesis of CCR5 to show that substitutions of the negatively charged aspartic acid residues at positions 2 and 11 (D2A and D11A) and a glutamic acid residue at position 18 (E18A), individually or in combination, impair or abolish CCR5-mediated HIV-1 entry for the ADA and JR-FL M-tropic strains and the DH123 dual-tropic strain. These mutations also impair Env-mediated membrane fusion and the gp120-CCR5 interaction. Of these three residues, only D11 is necessary for CC-chemokine-mediated inhibition of HIV-1 entry, which is, however, also dependent on other extracellular CCR5 residues. Thus, the gp120 and CC-chemokine binding sites on CCR5 are only partially overlapping, and the former site requires negatively charged residues in the amino-terminal CCR5 domain.

CD4 and 7-transmembrane (7TM) proteins of the chemokine receptor family directly interact with the human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins to initiate viral entry into target cells (1, 11, 15, 17, 20, 22). The tropism of different viral strains can now be explained broadly by their coreceptor specificity; CCR5 is used by macrophage (M)-tropic strains (1, 11, 15, 17, 20), and CXCR4 is used by T-cell-tropic and T-cell line-adapted (TCLA) strains (22). Most primary isolates are capable of using both coreceptors and are therefore dual tropic, although CXCR4 usage acquisition correlates with advanced disease progression (14, 44). Recently, a number of other 7TM proteins belonging, or closely related, to the chemokine receptor family have been shown to function as coreceptors for certain strains of HIV and/or simian immunodeficiency virus (SIV) (11, 16, 17, 21, 25, 36). However, CCR5 plays a key role in HIV-1 transmission since individuals homozygous for a 32-bp deletion in the CCR5 gene are resistant to infection and lymphocytes from these individuals do not support entry of M-tropic strains (27, 32, 40). The roles of the other coreceptors in HIV-1 transmission and pathogenesis remain to be established, but this makes them no less interesting from a molecular perspective. Comparative studies of CCR5 and other coreceptors capable of interacting with M-tropic strains are likely to yield important clues about the structural determinants necessary for coreceptor activity. How interactions among gp120, CD4, and CCR5 trigger fusion of the viral and cellular membranes is far from established, but a number of studies have begun to shed light on the subject.

Proof of a direct interaction between gp120 and CCR5 comes from experiments showing that soluble gp120 from M-tropic strains, but not from TCLA strains, competes for CCR5 binding with MIP-1β, one of the chemokine ligands of CCR5 (45, 48). Furthermore, M-tropic gp120 lacking the V3 loop does not block MIP-1β binding, suggesting that this tropism-determining domain influences the gp120-CCR5 interaction (45, 48). Whether there is direct contact between the V3 loop and the coreceptor remains to be determined, but it is likely that other, more conserved regions of gp120 are also part of the CCR5 binding site. The gp120-CCR5 interaction is enhanced by CD4 binding (45, 48), and there is also evidence for an association of the D1D2 domains of CD4 with CCR5 (48). Thus, some major elements involved in the formation of the gp120-CD4-CCR5 complex have already been defined. However, much remains obscure about what elements of CCR5 are necessary for its coreceptor function.

A number of reports have described the ability of chimeras made between CCR5 and related receptors to mediate HIV-1 entry and/or fusion (2, 4, 33, 38). Murine CCR5 (mCCR5) is a nonfunctional coreceptor despite having 82% amino acid homology with its human counterpart (2, 4, 33). Human CCR2b only functions as a coreceptor for the dual-tropic 89.6 isolate (17). Replacing any one domain of human CCR5 (hCCR5) by the mCCR5 counterpart does not significantly impair coreceptor function yet no single domain of hCCR5 can impart wild-type activity on mCCR5, although some domain substitutions, especially in combination, can confer partial activity (2, 4, 33). Similarly, no single domain of CCR2b can knock out CCR5 coreceptor function, but when the amino terminus of CCR5 is grafted onto CCR2b a functional coreceptor is created (2, 38). Perhaps the most important conclusion that can be drawn from all of these observations is that multiple extracellular domains of CCR5 are involved, directly or indirectly, in its coreceptor function in the context of a chimeric receptor. Furthermore, different viral isolates have different requirements for the four

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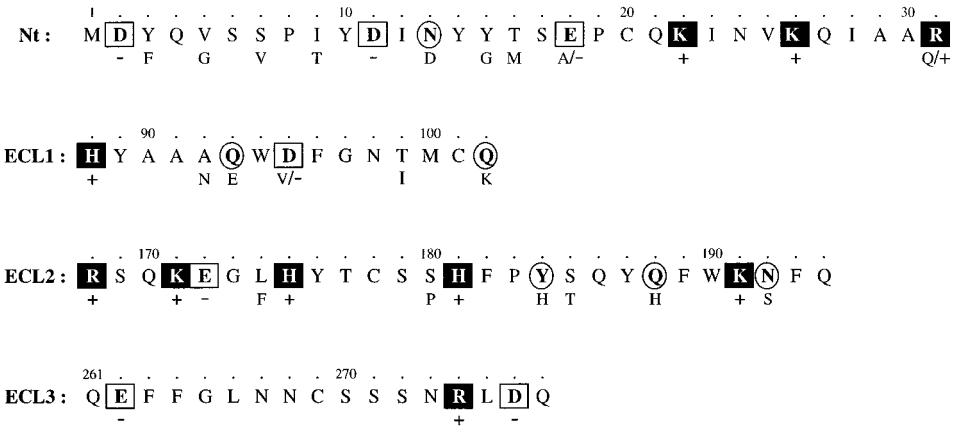

FIG. 1. Mutagenesis of the predicted four extracellular domains of CCR5. The amino acid sequences of the Nt region and three ECL (ECL1 to ECL3) of hCCR5 to hCCR3 are indicated. The polarity (+ or −) of charged residues is indicated below the main sequence, as are the identities of residues which differ in mCCR5. hCCR5 residues with negatively charged (white squares) and positively charged (black squares) side chains, and residues whose charges differed in mCCR5 (white circles), were all modified to alanine by PCR or site-directed mutagenesis. Fidelity was confirmed by sequencing both strands of the entire CCR5 coding region. In some cases, double mutants, K171A/E172A, K191A/N192A, and R274A/D276A, were made to preserve the overall net charge of their domain. The Nt double and triple mutants D2A/D11A and D2A/D11A/E18A were based on initial results with single-residue mutants.

extracellular regions of CCR5 (4, 38). To obtain more specific knowledge of which CCR5 domains are necessary for viral entry, we have studied the role of selected amino acid residues, rather than large segments of the molecule.

One of the most informative approaches to studying the functional topography of proteins is alanine-scanning mutagenesis. To identify residues of CCR5 involved in gp120 binding and HIV-1 entry, we have used this technique to alter negatively (D, E) or positively (K, R, H) charged residues in the amino terminus (Nt) and three extracellular loops (ECL1 to ECL3) (Fig. 1). Residues that differed between hCCR5 and its murine counterpart were also mutated whenever the difference involved a charge change (2, 4, 33) (Fig. 1). We chose this approach because extracellular domains, and especially the amino termini of other 7TM receptors, have been previously implicated in binding of peptide ligands (12, 29, 31, 42) and charged residues are involved in the interaction of CXCR2 with interleukin 8 (23). In all, 17 single, 4 double, and 1 triple mutants were studied. We tested their abilities (i) to perform as coreceptors for three different viral isolates, two M tropic and one dual tropic, (ii) to induce cell-cell fusion, and (iii) to support viral entry in the presence of MIP-1α, MIP-1β, or RANTES. Those mutants which were most impaired for entry and fusion were also tested for their ability to bind gp120 (JR-FL), by using a CCR5 monoclonal antibody (MAb) competition assay.

MATERIALS AND METHODS

Immunoblot analysis of CCR5 expression in whole-cell extracts and plasma membrane extracts. Lipofected U87MG-CD4 cells (10) from a 60-mm-diameter tissue culture plate were resuspended in a solution containing 1% sodium dodecyl maltoside, 10 mM Tris-HCl (pH 6.8), 50 mM NaCl, 1 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg of leupeptin/ml, 10 μg of aprotinin/ml, 0.7 μg of pepstatin/ml, and 10 mM EDTA. The suspension was incubated at 4°C for 30 min, and the supernatant fraction was collected after centrifugation. Alternatively, the cell pellet was resuspended in cold hypotonic buffer (1 mM Tris-HCl [pH 6.8], 0.1 mM PMSF, 5 μg of leupeptin/ml, 10 μg of aprotinin/ml, 0.7 μg of pepstatin/ml, and 10 mM EDTA) and forced three times through a 26-gauge needle. The lysate was layered onto a 36% (wt/wt) sucrose solution containing 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 1 mM $MgCl_2$, 10 mM EDTA, and 0.1 mM PMSF and centrifuged at 22,000 rpm for 20 min at 4°C. The interface band containing the plasma membrane fraction was collected, washed twice with the hypotonic buffer, and solubilized in the lysis buffer, like the whole-cell pellet.

Total protein concentrations were determined with the Bio-Rad DC Protein Assay. Protein (15 μg total for whole-cell extracts and 6 μg total for plasma membrane extracts) was then fractionated, without prior boiling, on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred to Immobilon-P membranes (Millipore) and probed for CCR5 with rabbit anti-hemagglutinin (HA)-tag antibody (1:500 dilution; Berkeley Antibody Company) and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G ($1:10^4$ dilution; Amersham), followed by incubation with chemifluorescent substrate (Vistra ECF; Amersham). Relative fluorescence emission (rfe) of immunoreactive bands, excited at 450 nm, was detected on a laser-based scanner (Molecular Dynamics Storm 860).

Lipofections and reporter gene assays. Mutated cDNAs were subcloned into the pcDNA3.1 (Stratagene) expression vector. U87MG-CD4 and SCL1-CD4 cells (10) were incubated with Lipofectin (5 μg/ml) and mutant DNA (4 μg/ml) plus pSVlacZ (1 μg/ml) in OPTI-MEM (Gibco BRL) for 5 h at 37°C. The cells were infected 24 h later with NLluc-Env supernatants, containing 200 to 500 ng of p24/ml, for 2 h at 37°C. For CC chemokine blocking of HIV-1 entry, 2 μg of MIP-1α, MIP-1β, or RANTES (R&D Systems)/ml was added simultaneously with HIV-1 (50 to 100 ng of p24/ml) and maintained in the cultures for 12 h. Cell samples were treated with 100 μl of lysis buffer (Promega) 72 h after infection, and luciferase (luc) and β-galactosidase activities (optical density at 420 nm [OD_{420}]) were measured. The percent wild-type (wt) standardized luc activity is defined as [(mutant luc cps/wt luc cps) × (mutant OD_{420}/wt OD_{420}) × (mutant rfe/wt rfe)] × 100 (cps, counts per second). The relative percent inhibition by a CC chemokine for each mutant is defined as [1-(luc cps with chemokine/luc cps without chemokine)]/[1-(wt luc cps with chemokine/wt luc cps without chemokine)] × 100.

Competition between gp120 and 2D7 MAb for CCR5 binding. HeLa cells (2×10^6) were incubated for 5 h with Lipofectin (10 μg/ml) and the pCDM8-CD4 expression vector (3.75 μg/ml) plus wt or mutant CCR5 plasmids (1.25 μg/ml) in OPTI-MEM. The cells were then infected for 12 h with 2×10^7 PFU of vTF7 to boost CCR5 and CD4 expression (20, 22), detached with 2 mM EDTA in phosphate-buffered saline (PBS), and washed once with binding buffer (1% bovine serum albumin, 0.05% azide in PBS). Cells (10^6) were incubated for 1 h at 37°C with or without 10 μg of gp120 (JR-FL)/ml (45) before addition of phycoerythrin (PE)-labeled 2D7 MAb (200 ng/ml) (6, 50) for 30 min at 4°C. The cells were washed once with binding buffer and once with PBS, resuspended in 1% formaldehyde in PBS, and analyzed by fluorescence-activated cell sorting (FACS) to determine mean fluorescence intensity (mfi). Percent inhibition of 2D7-PE binding is defined as [1-(mfi with gp120/mfi without gp120)] × 100. CD4 expression was monitored by staining with Leu3A and varied by no more than ± 10% between samples.

RESULTS

Cell surface expression of CCR5 mutants. All CCR5 molecules used in this study had a nine-residue HA tag as a carboxy-terminal extension to allow detection by Western blotting (3). This system presents an advantage over detection by anti-CCR5 antibodies since it does not depend on epitopes in CCR5 itself, which may be altered by mutagenesis. How-

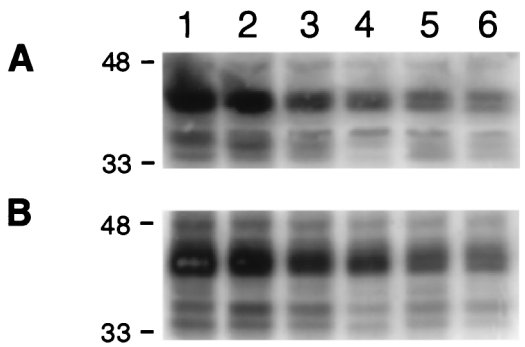

FIG. 2. Expression of CCR5 coreceptors in lipofected U87MG-CD4 cells. CCR5 proteins in detergent lysates of whole cells (A) or plasma membranes (B) were detected and compared by Western blotting with anti-HA-tag antibodies. Mature receptor proteins migrate with an apparent molecular mass of approximately 40 kDa. Lanes: 1, wt CCR5; 2, D2A; 3, D11A; 4, E18A; 5, D2AD11A; 6, D2AD11AE18A. The amount of mutant receptor relative to that of CCR5 was quantitated by comparing the integrated fluorescence intensities of 42-kDa bands in respective lanes. Molecular mass markers (in kilodaltons) are at left.

ever, it was important to show that protein expression levels detected in whole-cell extracts, which we used to standardize our luciferase readouts, correspond to cell surface expression levels. We therefore performed Western blots to compare coreceptor levels in whole-cell extracts and plasma membrane extracts of U87MG-CD4 cells transiently lipofected with wild-type CCR5 or the amino-terminal (Nt) mutants D2A, D11A, or E18A, the double mutant D2A/D11A, or the triple mutant D2A/D11A/E18A (Fig. 2). CCR5 expression patterns detected in whole-cell extracts were indeed identical to those obtained in plasma membrane extracts, and mutant protein levels ranged between 20 and 100% of that of the wt protein (Fig. 2). Protein levels for all of the other mutants were determined only in whole-cell extracts and ranged between 30 and 100% of that of the wt protein (data not shown). The relationship between wt CCR5 expression levels and HIV-1 entry efficiency was determined to be linear over the relevant 10-fold range (data not shown).

Coreceptor function of CCR5 mutants in U87MG-CD4 cells. The wt and mutant CCR5 proteins were transiently expressed in both U87MG-CD4 and SCL1-CD4 cells, and their abilities to support entry mediated by HIV-1 envelope glycoproteins were determined using an *env*-complementation assay with a luciferase readout (9, 11, 15, 17, 20). These nonlymphoid human cell lines were chosen because they lack CCR5, CCR3, and CXCR4 and therefore resist infection by HIV-1 in the absence of a transfected coreceptor (15, 16, 20). (A few exceptional HIV-1 strains will enter U87MG-CD4 cells via gpr1 [16], but we did not use them.) Almost identical results were obtained with both cell lines. Two M-tropic viruses, ADA and JR-FL, that use CCR5 but not CXCR4 (1, 15, 20, 22), and one dual-tropic virus, DH123, that uses both CCR5 and CXCR4 equally well (19, 43), were used to test whether the mutant CCR5 proteins could support HIV-1 entry. The level of expression of each transfected CCR5 mutant was assessed by Western blotting and taken into account when determining coreceptor efficiency.

Of the 17 single mutations that we made, only 3 had a significant inhibitory effect on the coreceptor function of CCR5 (Fig. 3). These were D2A, D11A, and E18A, all located in the N domain of CCR5 (Fig. 1). The E18A substitution alone was sufficient to reduce CCR5 function by 15- to 20-fold. The double mutant D2A/D11A was less active than either of the single mutants (D2A or D11A), and the triple mutant (D2A/D11A/E18A) was almost completely inactive (>50-fold reduction in entry compared to wt; raw luc cps values ranged from 5×10^5 to 2×10^6 for wt CCR5) (Fig. 3a). None of the other substitutions significantly affected CCR5 function in this assay (Fig. 3b and c). Similar results were obtained with both M-tropic envelope glycoproteins. The only difference noted with the dual-tropic DH123 envelope was a significantly increased sensitivity to the D11A and R31A substitutions (Fig. 3a and b).

Cell-cell fusion induced by CCR5 mutants. To study the effects of the D2A, D11A, and E18A substitutions in an independent assay of HIV-1 Env-mediated function, we used a membrane fusion assay in which HeLa cells stably expressing the JR-FL *env* gene are mixed with HeLa-CD4 cells transiently transfected with wt or mutant CCR5 (20, 26). The two cell types are labeled with different fluorescent probes, and fusion is monitored by resonance energy transfer (RET), which occurs only when the two dyes are present in the same membrane (20, 26). We tested the D2A, D11A, and E18A single mutants and the double and triple mutants in comparison to wt CCR5 by RET assay (Fig. 4). Each mutant had a phenotype in this fusion assay similar to what was observed in the viral entry assay (cf. Fig. 2a and 3); the E18A and the double and triple mutants were completely unable to support Env-mediated membrane fusion. However, when we boosted the expression of coreceptors by about 100-fold by using the vaccinia virus-T7 polymerase (vTF7pol) system (1, 17, 20, 22), each of the CCR5 mutants was able to support membrane fusion, although less efficiently than the wt protein (Fig. 4). We noted previously that CCR5 overexpression abolished the ability of its CC-chemokine ligands to inhibit membrane fusion, suggesting that some phenotypic changes can be missed if CCR5 expression is too high (20). The results with the vTF7pol system do, however, show that even the triple mutant (D2A/D11A/E18A) is not completely inert as a coreceptor, just very strongly impaired.

Inhibition by CC-chemokines of mutant CCR5 coreceptor function. We next tested whether the CCR5 mutants that supported HIV-1 entry were sensitive to the inhibitory effects of the CC-chemokine ligands of CCR5: MIP-1α, MIP-1β, and RANTES (Table 1) (13, 37, 39). (Note that the D2A, D11A, and E18A mutants did support enough entry for their sensitivity to inhibition to be determined. However, this was not true of the Nt double and triple mutants. With U87MG-CD4 cells, as with other nonlymphoid cells, the CC chemokines do not completely block HIV-1 infection, and high concentrations are needed to obtain an inhibitory effect (15, 20, 41, 45). Thus, we compared the degree of inhibition achieved by the CC-chemokines on the mutant and wt CCR5 receptors. This was in the range of 40 to 60%, depending on the particular ligand, with the rank order for potency being RANTES > MIP-1β > MIP-1α, as it is in $CD4^+$ T cells (46). The following mutants were relatively insensitive to the action of one or more of the CC-chemokines: D11A, K22A, R31A (Nt); H181A, Y184A, K171A/E172A, K191A/N192A (ECL2); R274A/D276A (ECL3). Of these, only D11A was impaired for both HIV-1 entry and CC-chemokine inhibition of entry. Amino acid substitutions at certain positions (mostly in the Nt domain and ECL2) do not, therefore, affect the HIV-1 coreceptor function of CCR5 but do affect CC-chemokine-mediated inhibition of this process (Table 1). There are also minor differences in CCR5 amino acid usage among the three chemokines. The mechanism by which the inhibitory substitutions affect the action of the CC-chemokines has not yet been determined. However, the simplest interpretation is that the CC-chemokine binding site and the HIV-1 interactive site on CCR5 are not

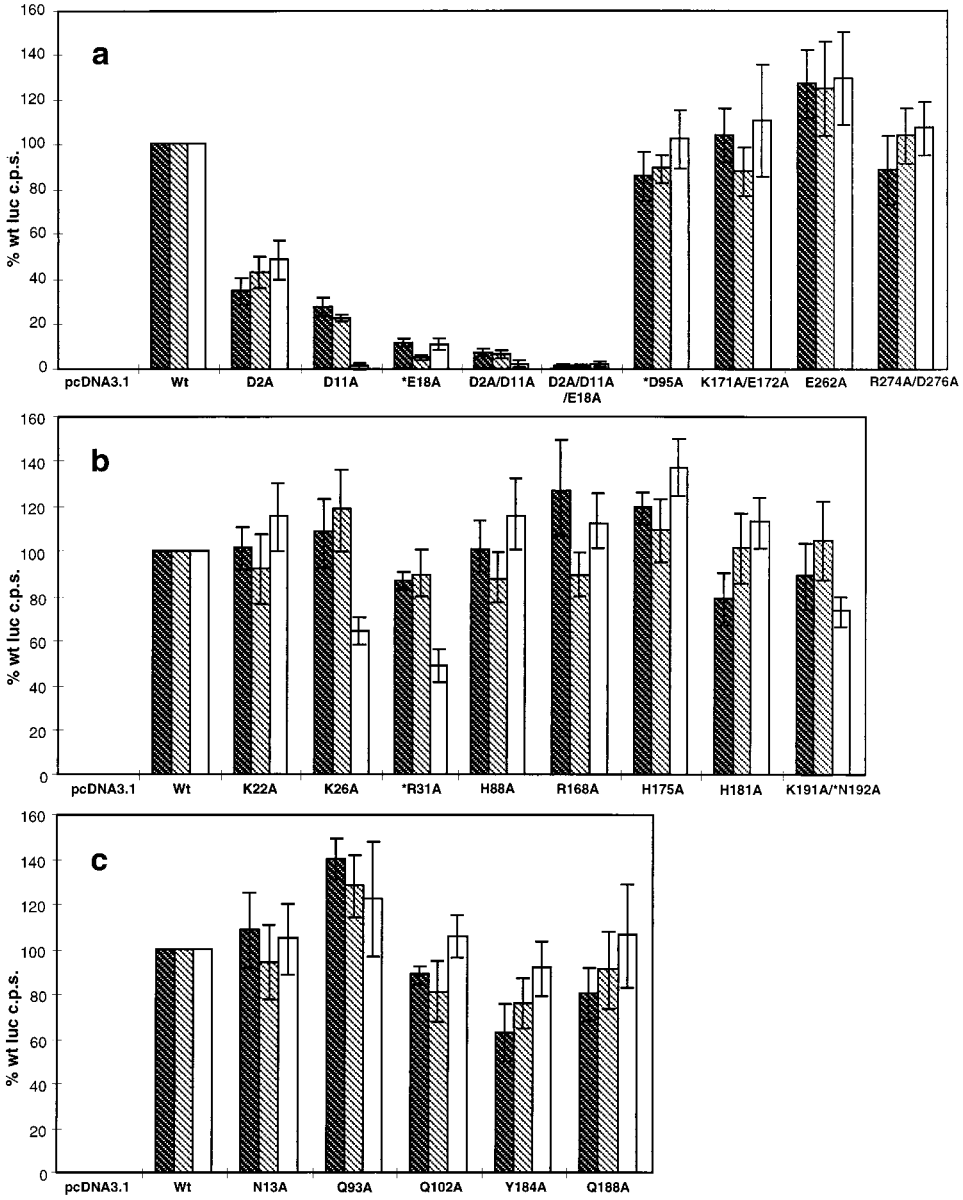

FIG. 3. HIV-1 coreceptor function of CCR5 mutants. Substitutions in negatively charged residues (a), positively charged residues (b), and selected murine residues differing from the human sequence (c) were tested for their effects on HIV-1 entry. U87MG-CD4 cells were transiently lipofected with CCR5 mutants and then infected with NLluc-ADA (dark hatched bars), NLluc-JR-FL (light hatched bars), or NLluc-DH123 (white bars) luc-expressing chimeric viruses. Luc activity (luc cps) was measured 72 h postinfection and standardized for lipofection efficiency and receptor expression levels. The coreceptor activity of each mutant designated on the *x* axis is expressed as a percentage of the wt coreceptor activity (100%). Values are means ± standard deviation (indicated by error bars) of three independent experiments, each performed in quadruplicate. The asterisks indicate that the amino acids are also different in mCCR5. Similar results (not shown) were obtained with SCL1-CD4 cells.

identical and that certain substitutions in ECL2 and ECL3 affect only the CC-chemokine binding site.

Nt mutants D2, D11, and E18 affect gp120 binding to CCR5. To understand how the Nt substitutions affect the HIV-1 coreceptor function of CCR5, we determined whether they affected gp120 binding. We were unable to measure the binding of labeled gp120 to CCR5 directly, because the level of CCR5 expression on transiently transfected cells was too low to obtain a reproducible signal in any of several binding assays tested. We therefore used a competition assay, in which the ability of gp120 (JR-FL) (45) to inhibit the binding of a PE-labeled CCR5-specific MAb (2D7-PE) (6, 48, 49) was measured. The epitope for this MAb is located within ECL2, and we found that it was able to bind efficiently to HeLa cells cotransfected with CD4 and the CCR5 Nt mutants and infected with vTF7. These experiments also provided us with additional evidence that our CCR5 mutants were expressed at the cell surface. The relative expression levels obtained by FACS were comparable to those obtained with Western blotting (data not shown).

Independent studies have shown that 2D7 inhibits the binding of ^{125}I-labeled gp120 to CCR5 on the murine L1.2 cell line (49), which overexpresses CCR5 to an extent that permits the detection of gp120 binding (48, 49). Here we show that the binding of 2D7-PE to wt CCR5 was strongly inhibited (70%) by prior addition of gp120, indicating that the interaction of

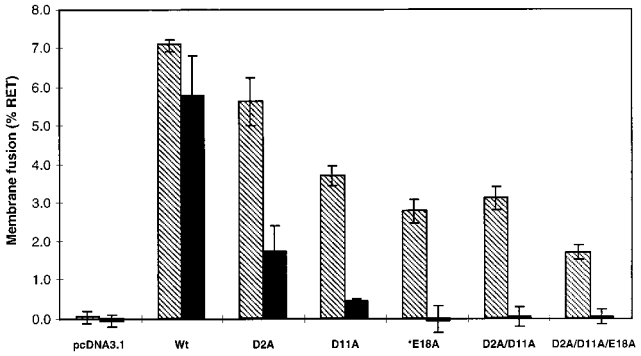

FIG. 4. Membrane fusion activity of CCR5 Nt mutants. HeLa-CD4 cells were lipofected with the Nt mutants indicated (or the pcDNA3.1 negative control plasmid) and tested 12 h later for their ability to fuse with HeLa cells expressing the JR-FL *env* gene (black bars). The vTF7pol system was used to enhance coreceptor expression (hatched bars). The extent of cell-cell fusion was determined by RET assay. The % RET values shown are the means ± standard deviations (indicated by error bars) of three independent experiments, each performed in duplicate. *E18A, the amino acid is also different in CCR5.

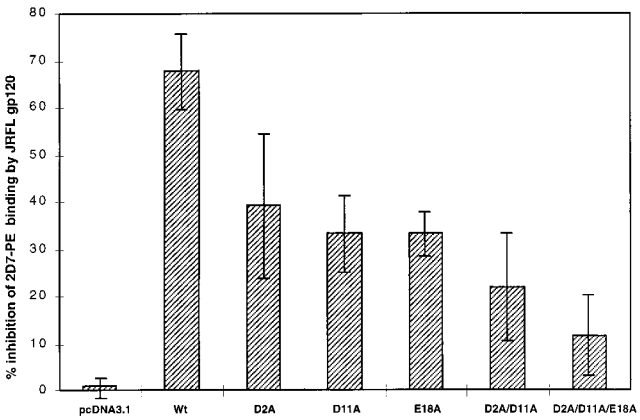

FIG. 5. Competition between gp120 and CCR5 MAb 2D7 for CCR5 binding. HeLa cells cotransfected with CD4 and either wt or mutant CCR5, and infected with vTF7pol to enhance receptor expression, were preincubated with or without 10 μg of gp120/ml (JR-FL) before addition of the PE-labeled 2D7 MAb (200 ng/ml) and FACS analysis to determine mfi.

gp120 and 2D7 with the receptor is mutually exclusive (Fig. 5). However, gp120 only partially inhibited (40%) the binding of 2D7-PE to the D2A, D11A, and E18A mutants and was almost completely ineffective at blocking 2D7-PE binding to the double and triple Nt mutants (25% and 15% inhibition, respectively) (Fig. 5). Of note, those mutants most impaired for HIV-1 entry (Fig. 3) were also the ones for which 2D7-PE binding was least sensitive to gp120 inhibition (Fig. 5). The most probable explanation of this result is that gp120 binds to the wt CCR5 molecule in such a way as to sterically hinder the binding of 2D7-PE to ECL2 and that gp120 binds poorly to the Nt mutants. A less likely possibility is that gp120 does bind efficiently to the Nt mutants but in an unusual orientation in which it is less able to inhibit 2D7-PE binding to ECL2. In the latter case, the geometry of interdomain interactions in CCR5 has been altered by the Nt substitutions that impair CCR5 coreceptor function.

TABLE 1. Inhibition of coreceptor function by CC chemokines[a]

CCR5 region	Mutant	Mean relative inhibition (%) of coreceptor function with[b]:		
		MIP-1α	MIP-1β	RANTES
Nt	D2A	81	97	85
	D11A	**10**	**41**	**7**
	N13A	121	93	92
	E18A	100	100	62
	K22A	**19**	**12**	**−11**
	K26A	100	97	100
	R31A	**−5**	**2**	**−16**
ECL1	H88A	98	95	99
	Q93A	88	97	114
	D95A	107	112	121
	Q102A	107	98	93
ECL2	R168A	90	88	96
	K171A/E172A	**21**	97	107
	H175A	119	100	95
	H181A	**29**	53	**39**
	Y184A	102	68	**36**
	Q188A	95	75	51
	K191A/N192A	**14**	15	**18**
ECL3	E262A	100	102	100
	R274A/D276A	**48**	**36**	**33**

[a] U87MG-CD4 cells were transiently lipofected with wt or mutant CCR5 and then infected with NLluc–JR-FL in the presence or absence of 2 μg of MIP-1α, MIP-1β, or RANTES/ml. Luc activity was measured 72 h later.

[b] Values are relative to inhibition by wt CCR5 (set at 100%) and are means of three independent experiments, each performed in quadruplicate. Values <50% of that observed with wt are in boldface.

DISCUSSION

In this study, we have identified point substitutions of three negatively charged residues in the Nt domain of CCR5 that, in combination, severely impair its HIV-1 coreceptor function. These Nt substitutions affect the ability of gp120 to bind correctly to CCR5, probably by reducing the affinity of the gp120-CCR5 interaction. This may well be sufficient to account for the coreceptor-impaired phenotype. The loss of membrane fusion capability caused by the Nt substitutions in CCR5 can be partially compensated for by overexpressing the mutant coreceptors, presumably because an increase in the number of low affinity coreceptors compensates for their reduced affinity. This could enable a successful gp120-CCR5 interaction to occur sufficiently rapidly to be compatible with the conformational changes in the envelope glycoproteins that initiate membrane fusion (5, 8, 47). Only one of these Nt substitutions, D11A, also interferes with CC-chemokine inhibition of coreceptor function. In contrast to the gp120-binding site, the CC-chemokine binding site on CCR5 is dependent on residues in both the amino terminus and the ECLs (notably, but not exclusively, ECL2). Thus, although they are not identical, there is some overlap between the gp120 and CC-chemokine binding sites, a conclusion consistent with studies showing that HIV-1 and SIV gp120 binding inhibit that of MIP-1β (24, 45, 48).

The gp120 binding site on CCR5 depends on three negatively charged residues in the Nt region. It will be important to determine whether these residues interact directly or indirectly with positively charged amino acids in gp120, be they in the V3 loop and/or elsewhere. We do not yet know which other Nt residues also contribute to the gp120 binding site, and whether residues in other regions of CCR5 are also involved. Previous studies using chimeric receptors or deletion mutants pointed to the importance of the CCR5 and CXCR4 amino termini for coreceptor function (2, 4, 7, 33–35, 38). Deletions beyond P8

and down to T16 of the CCR5 amino terminus seriously impair the ability of 89.6 Env but not JR-FL Env to induce fusion via these truncated coreceptors (38). A more recent study shows that the triple mutant D11A/K197A/D276A impairs cell-cell fusion driven by the dual-tropic 89.6 Env but not by the M-tropic JR-FL Env (18). However, there was little or no effect of the D2A, D11A, and E18A single mutants on CD4-Env-induced fusion in this system. We believe that cell-cell fusion assays in which expression of envelope glycoproteins, CD4, or the coreceptors is driven by vTF7pol may not always detect phenotypic changes that can be compensated for by overexpression of the components of the fusion machinery (see Fig. 4). We have also noted that in the luc *env*-complementation assay, NLluc-89.6 enters U87MG-CD4-CCR5 (24) cells about 100-fold less efficiently than NLluc–JR-FL does (19). Hence the inefficient interaction of 89.6 Env with CCR5 may be more sensitive to impairment of CCR5 function than the interaction mediated by JR-FL Env.

Despite the apparent importance of the Nt region of CCR5 for gp120 binding and HIV-1 entry, replacing the entire Nt of CCR1 or CXCR4 with that of CCR5 does not yield functional coreceptors (19). In addition, replacing the CCR5 Nt by that of CXCR4 abolishes coreceptor function for M-tropic strains, while not conferring specificity for TCLA strains (19). Thus, our limited studies of chimeric receptors also indicate that the CCR5 Nt is important but not sufficient for coreceptor function. The gp120 binding site on CCR5 is probably made of multiple determinants, dispersed over some or all of the extracellular domains. It is possible that different receptors possess only some of these determinants, which can add up to a (partially) functional binding site in certain chimeras (2, 4, 18, 33, 38). For example, the mCCR5 amino terminus, which lacks E18 (but has an aspartic acid at position 13), is functional in the context of the MHHH chimera (2, 4, 33). However, the possibility should not be discounted that alterations in the ECLs of receptor chimeras may indirectly affect the orientation of the CCR5 Nt and hence its ability to interact correctly with gp120.

Other retroviruses use multiple-membrane-spanning proteins as receptors (30). It is therefore likely that some feature of these molecules, such as their proximity to membrane lipids, renders them suitable for inducing membrane fusion, possibly through a common mechanism. Although it is not yet clear whether the efficiencies with which one viral strain interacts with divergent coreceptors are always comparable, these observations do suggest that there is a considerable degree of tolerance for variations in coreceptor sequence. Perhaps, among functional HIV-1 coreceptors, there is conservation of a common framework with which gp120 can interact, superimposed on a more variable structure. Defining the binding sites on the other coreceptors and assessing their similarity to the binding sites on CCR5 and CXCR4 may shed light on this issue. (It should be noted, however, that the ability of a coreceptor to interact with CD4 as well as gp120 might also be crucial to the efficiency with which it is used by HIV-1.) The coreceptor binding site on gp120 may also have variable components superimposed on a conserved framework and may be subtly different between isolates. There may even be a continuum of HIV-1 phenotypes, each one corresponding to a slightly different fit of the gp120-coreceptor complex. Recent studies showing that dual-tropic and TCLA strains use different regions of CXCR4 illustrate this point (7, 28, 34). In light of this, it was unexpected that the dual-tropic DH123 isolate was similar to the M-tropic isolates ADA and JR-FL in its sensitivity to almost all of the substitutions we made in CCR5. However, we have recently obtained data showing more profound differences in the way M-tropic and dual-tropic viruses interact with this coreceptor (19).

A more detailed understanding of the binding sites on different coreceptors for viral envelopes will be required to define how HIV-1 uses these molecules for entry into its target cells. It is important to remember that the final result of the gp120-CD4-CCR5 interaction is membrane fusion. The mechanism by which bilayer-lipid mixing is induced by protein-protein interactions remains elusive, but HIV-1 offers a model that may shed light on this fundamental process of virology.

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